STAIN TECHNOLOGY

VOLUME 21 APRIL, 1946 NUMBER 2

PROGRESS IN THE STANDARDIZATION OF STAINS

RECENT ADVANCES

In recent editorials of this series (during 1944 and 1945), hints have been made to the effect that plans were under way which the Stain Commission hoped to bring to fruition after the war. Some of these plans represented projects already well started while others were still for the future. It seems well now to try to size up the situation and to see how well the plans are maturing.

PUBLICATIONS

The fifth edition of Biological Stains, which has been requiring considerable work for over a year, and which has been delayed time and time again by difficulties with which the printing industry is faced these days, is now on the press; it is hoped that its publication will not be very much behind that of this issue of STAIN TECHNOLOGY. It is unfortunate that the fourth edition should have gone out of print during the war, at a time when stains were in such demand; but that could not be prevented, and the very situation which has caused the demands for stains has also caused the delays in getting any kind of work (e.g. printing) done on schedule. It is hoped, however, that the fifth edition will be enough better than the fourth to make up for the delay.

Staining Procedures is now complete, with the publication of its tenth leaflet (an index) within the last few months. Although this loose-leaf manual is not so well known as the Commission's older publications, there has been considerable demand for it, in fact a rather surprising demand, considering the small amount of advertising that has been given the publication.

A 20-year index to Stain Technology has been prepared, which should be ready for publication in a month or two. This cannot be promised for any definite time, as the typesetting is not yet complete and no date for its going to press has yet been set. Previously it has been the custom to print a 5-year index at the end of every fifth volume and to include it with the first issue of the following volume. The 5-year index has been omitted after volume 20, because of the contemplated 20-year index; the latter will be too

long to include as part of a regular issue. It will have to be sold as a separate publication.

NEW ASSAY METHODS

The testing of samples submitted for certification has always been of three sorts: tests for actual performance; chemical assay to determine dye strength; spectrophotometric tests to learn whether or not the sample is true to type.

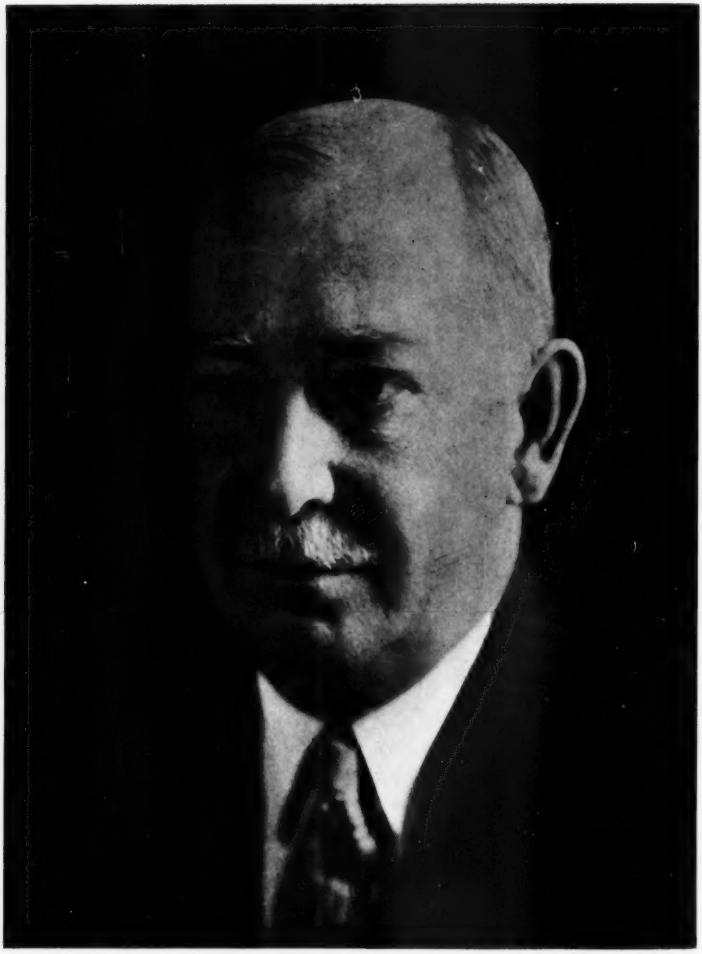
The tests for actual performance are being improved all the time in little particulars; at their best, however, the tests selected do not represent an ideal method of assay, and it is hoped that some day chemical and spectrophotometric methods will replace them. Until such time, however, they must continue to be employed; in fact, it is necessary to lay more stress on them than on chemical methods, which do not always correlate with performance. Imperfect as they are, however, they were regarded as of sufficient significance to be accepted by Army and Navy, during the war, as a basis for the approval of stains for the armed forces. The methods now in use are to be found in an appendix of the fifth edition of Biological Stains.

Chemical assay methods, primarily titration with titanium chloride to indicate dye strength, are also being perfected, little by little. They have been drawn up now in sufficiently definite form so that it has proved possible to transfer the testing from one laboratory to another without experiencing serious difficulty. These methods also are included in the appendix of Biological Stains.

Spectrophotometric methods are not so well standardized. They are given in the same appendix; but the methods there given are subject to revision at the present time due to the availability of new types of spectrophotometers more precise than the older instruments. Fortunately arrangements have been made to review these methods at Geneva, and it is expected that in a short time, distinctly improved procedures for spectrophotometric assay will be available. The first of a series of papers on this subject is given in this issue of STAIN TECHNOLOGY (p. 49).

RESEARCH WORK

The Stain Commission has begun a new departure in making a grant to Northwestern University for the continuance of research work in neurological methods formerly carried on by Dr. H. A. Davenport, but which was interrupted by his service in the war. Readers of STAIN TECHNOLOGY will recall his papers that have appeared from time to time in these pages. It is gratifying that the Stain Commission is now in a position to enable him to continue the work again.—H. J. CONN.



CLARENCE ERWIN McCLUNG
1870–1946

CLARENCE ERWIN McCLUNG

In Memoriam

In the sudden death of Clarence Erwin McClung on January 17, 1946, the field of microtechnic lost a distinguished devotee.

C. E. McClung was born in Clayton, California, April 5, 1870. His father was a civil and mining engineer and his mother the daughter of a physician. He grew up in the state of Kansas where he received his education and embarked upon his professional career.

As a boy he displayed a marked interest in chemistry, and after working in his uncle's drugstore, enrolled in the School of Pharmacy at the University of Kansas, receiving the Ph. G. degree in 1892. After teaching chemistry and pharmacy for a year, he entered the college and continued in graduate work, receiving the A. B. degree in 1896, the A. M. in 1898 and the Ph. D. in 1902.

Dr. McClung early developed a dominating interest in the microscope and in microscopical technic, and he entered Professor S. W. Williston's class in animal histology. Here he displayed so much ability and interest that, half-way through the course, Dr. Williston asked young McClung to take charge. In this way he became embarked upon a career in zoology. His work on chromosomes brought him world renown. His discovery of the chromosomal mechanism of sex-determination in insects was a landmark in the development of an understanding of the relations between chromosomes and body characteristics.

From the beginning of his career Dr. McClung was interested in the microscope and the technics employed in its use. As early as 1892 he published a paper on glycerin-mounting and a year later one on "The use of stains in microscopy". Other publications on technic appeared from time to time culminating in the "Handbook of Microscopical Technique" which he prepared with the help of a group of collaborators. First published in 1929, it was followed by a second enlarged edition in 1937. Dr. McClung was mechanically inclined and he devised improvements in microscope design, so that eventually a "McClung Model" was produced by one of the microscope manufacturing companies. When Dr. McClung became director of the zoological laboratory at the University of Pennsylvania in 1912, he took charge of the course in microscopical technic and directed it until his retirement in 1940. In this course he stressed the fundamental principles upon which the technical methods are based.

Dr. McClung's keen intellect, genial personality and administrative ability brought him many responsibilities both inside and outside the universities which he served. At the University of Kansas he was

made head of the department of zoology in 1901, only five years after receiving the A. B. degree, and served on many administrative committees. At the University of Pennsylvania he interested himself in curricular reforms and in improvements in facilities and opportunities for graduate study and research. Here also he was chairman or member of many important committees. At the Marine Biological Laboratory, Woods Hole, Mass., where he and his family spent most of their summers after 1912, he became trustee in 1913, member of the investigative staff in 1914, and served on such important committees as that to secure endowment, the committee on plans for the new permanent buildings, the library committee and the executive committee.

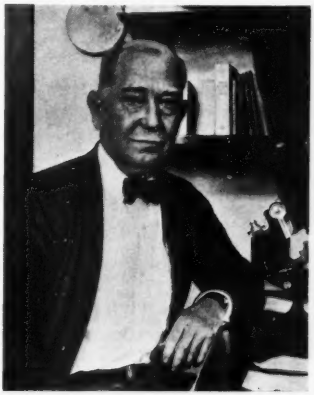
In 1917 Dr. McClung was made chairman of a zoology committee in the Division of Biology and Medicine of the National Research Council. In 1919 he became the first chairman of the new Division of Biology and Agriculture which he helped to organize. While in this position he initiated plans for a comprehensive abstracting service in the field of biology and for methods of improving and standardizing biological stains. Out of these efforts arose the formation of the Union of American Biological Societies of which he was president from 1922 to 1930. This organization, with others, sponsored Biological Abstracts, and he was president of its Board of Trustees from 1925 to 1938. He was also editor of its section on animal cytology from the beginning.

In regard to the project to improve and standardize biological stains, Dr. H. J. Conn, President of the Biological Stain Commission, has this to say, in correspondence:

"It was about this time that my own interest in improving and standardizing American biological stains began. These investigations were begun in 1920 or 1921 and were at that time confined purely to the bacteriological uses of those few stains which bacteriologists used in quantity. At the same time Dr. McClung, unknown to me, was becoming interested in the same problem from the cytological angle. The stain which had given him the most trouble was hematoxylin and his method of attacking the problem was by attempting the laboratory manufacture of this dye and in this way learning what was really at fault in the American commercial product. This was obviously an entirely different method of attack from that which I was pursuing from the bacteriological angle—namely, the coöperative investigation of a considerable series of commercial samples to learn which were the more satisfactory.

"As Director of one of the divisions of the National Research Council, Dr. McClung was naturally interested in seeing the problem attacked

on a wider basis as a coöperative project under Research Council auspices. When he learned that bacteriological investigations on the subject were already under way, that equally difficult problems were being faced in other biological lines, and finally, that certain commercial men were just as eager to see the subject standardized, it seemed quite an obvious move to call a conference of all those interested in the subject. He may have been the first one to propose this conference, although before the step was actually taken, his term of office in the National Research Council was over, and his successor, Dr. L. R. Jones, called the conference. Certainly Dr. McClung was at the meeting, partly as the retired Director of the Division and partly as a representative of zoological science, and he took a very active part in the discussion. The result of this conference need not be detailed here, except to say that the Biological Stain Commission was the eventual outgrowth of it."



At Woods Hole

As a man, Dr. McClung was known not only for his keen analytical mind, lively imagination and active sense of humor, but also for his natural simplicity and sympathetic friendliness. These qualities made him a delightful companion on almost any occasion. He was always ready to offer kindly counsel to the many associates and students who sought his advice, and his unselfish concern with the interests and welfare of those around him was proverbial.

The artistic side of his nature revealed itself in a liking for composition and literature, developed as an undergraduate; in his devotion to various forms of music and pictorial art; in his mastery of different methods of drawing as revealed by the expertly executed illustrations in his scientific papers; in his enthusiasm for photography, and an abiding interest in dramatics.

As a teacher, Dr. McClung sought to develop the innate capabilities of the individual. Even in the elementary course in zoology, which he taught at Kansas, he introduced "unknowns" to arouse the investigative interest which most young people have but which is all too often prevented from being exercised. By personal interviews with his students, he tried to learn the reasons for varying degrees of

success or failure and to stimulate efforts toward improvement. As editor of the Journal of Morphology from 1920 until his death, he labored to help zoologists, especially the younger ones, to an appreciation of the best methods of writing scientific papers.

Dr. McClung's abilities and accomplishments were recognized in many ways. He was president of the Central Branch of the American Society of Zoologists, 1910; president of the combined Society, 1914; president of the national society of Sigma Xi, 1917–21; vice-president of Section F (Zoology), A. A. A. S., 1926; president of the American Society of Naturalists, 1927; president of Beta Beta Beta Honorary Biological Fraternity, 1936 until his death, and was a member of the American Association of Anatomists, American Philosophical Society, the Kansas Academy of Sciences, the Washington Academy of Sciences, and National Academy of Sciences. In 1922, during the scientific meetings in Boston, a testimonial dinner was tendered to Dr. McClung, celebrating his 25 years as a teacher of zoology. In 1930 he represented the U. S. Government, the American Philosophical Society and the National Academy of Science at the International Congress of Biology at Montevideo. In 1933–34 he was visiting Professor at Keio University, Tokyo, Japan.

At the time of his retirement in 1940 Dr. McClung was made Emeritus Professor and awarded the honorary degree of Doctor of Science at the Bicentennial Commencement of the University of Pennsylvania. At a dinner held in his honor he was presented with a dedicatory volume of the Journal of Morphology to which 30 of his former students contributed papers; a "McClung Model" research microscope; and a bound volume containing more than 200 letters of greeting from friends throughout the world.

The year following his retirement was spent at the University of Illinois as acting head of the department of zoology. In 1941 the University of Kansas initiated the awarding of Distinguished Service citations and Dr. McClung was among the first group of alumni to be thus honored. In 1942 he was given the honorary degree of Doctor of Science by Franklin and Marshall College. In 1943, when Dr. Laurence Irving entered military service, Dr. McClung was invited to become acting head of the department of biology at Swarthmore College.

Dr. McClung's friends throughout the world now mourn the passing of a distinguished scientist, a great teacher and a true friend.

D. H. WENRICH, *Philadelphia, Pa.*

COMPARATIVE ABSORPTION READINGS OBTAINED WITH SPECTROPHOTOMETERS OF VARIOUS TYPES*

H. J. Conn and Elmer Stotz, *New York State Agricultural Experiment Station, Geneva, New York*

With the technical assistance of Mary A. Darrow.

One of the most important means of identifying a dye is by spectrophotometry. This method has been used as a criterion for judging the samples of stains submitted by manufacturers ever since the certification plan for biological stains was adopted. All specifications that have been drawn up for biological stains, such as those given in the 7th edition of the National Formulary, have included spectrophotometric requirements.

The principle of the method has been discussed in so many other places that it does not need to be repeated here. It is enough to explain that by means of the spectrophotometer, an absorption curve can be obtained which is characteristic for any dye and is seldom displayed by any other dye or mixture of any two dyes. The important points in regard to any such curve are the following: whether it shows just one or more than one maximum; the wave length (λ) at the point of greatest absorption; the width and shape of the absorption band, as indicated by the slope of the curve on each side of the maximum.

The initial work by the Stain Commission on the spectrophotometry of biological stains was performed with a visual type spectrophotometer of Bausch and Lomb manufacture, and until recently this instrument was used as a means of obtaining all standard values to which later analysis could be referred. As a matter of fact, when this work was begun (some 20 years ago), no other type of spectrophotometer was available.

Within recent years, photo-electric spectrophotometers have been perfected and are becoming very popular. They have several advantages: greater speed and precision; and freedom from personal error due to eye fatigue or to varied sensitivity of the eye to light in different parts of the spectrum. The simplest form of photoelectric instrument calls for visual reading of the dials; this requires some time, but much less than when the comparison of color intensity must also be made visually. Among the speediest of modern

*Journal paper 669, New York State Agricultural Experiment Station, Geneva, New York, March 6, 1946.

spectrophotometers is the General Electric recording instrument which automatically draws a graph showing the transmittance throughout the entire spectrum; a complete curve for any sample can be made in a very few minutes.

These different types of spectrophotometers differ from one another in another important respect: namely, the width of the slit through which the light from the prism is allowed to pass to the photometer.

TABLE 1. COMPARATIVE SPECTROPHOTOMETRIC DATA OBTAINED ON THE VISUAL (B & L) VS. THE PHOTOELECTRIC (BECKMAN) SPECTROPHOTOMETER.

Dye	Nat. Formulary standards		Comparative determinations			
	Wave lengths of points selected for ratio	Ratio tolerance	Cert. No. of sample tested	Absorption maximum (λ)	B & L Ratio	Beckman Ratio
Yellow Dyes, λ=435–445 mμ						
Auramin O*	470/490	.....	NAu–1	438	7.50	7.95
Martius yellow*	500/530	.....	NYm–3	445	21.3	...
Brown and Orange Dyes, λ=465–490 mμ						
Bismarck brown Y*	500/530	1.75–2.28	NN–11	470	1.77	1.94
Methyl orange*	500/530	0.96–0.98	NM–9	465	0.96	1.59
Orange II*	500/530	1.95–2.01	COb–2	490	2.01	1.96
Orange G*	500/530	4.40–5.80	NO–10	480,495	5.20	5.75
Red Dyes, λ=510–560 mμ						
Sudan III	500/530	1.02–1.13	NY–8	510	1.07	1.07
Congo red	500/530	1.19–1.21	NQ–12	510	1.19	1.17
Sudan IV	500/530	0.90–0.94	NZ–14	520	0.92	0.92
Eosin Y	505/535	0.82–1.01	LE–18	525	0.96	0.58
Eosin B	515/545	.....	NEb–10	525	1.66	1.36
Erythrosin B	515/545	1.04–1.55	NEr–8	525	1.09	1.32
Erie garnet B	500/530	.....	NEg–1	525	0.94	0.88
Ethyl eosin	515/545	0.85–1.02	NEe–3	535	0.89	0.57
Safranin O	515/545	0.98–1.08	NS–19	535	1.00	0.87
Neutral red	515/545	0.73–0.77	NX–12	ca. 545	0.75	0.72
Rose Bengal	540/570	.....	CRb–4	555	1.98	1.26
Phloxine B	530/560	0.95–1.50	NPh–11	550	1.07	0.70
Pararosanilin	530/560	1.12–1.17	NF–48	548	1.17	0.96
Fuchsin, acid	530/560	.....	NR–18	552	0.95	0.81
Rosanilin	530/560	0.87–0.91	CF–30	552	0.89	0.79
Pyronin Y	530/560	0.76–1.00	NP–10	552	0.87	0.65
Pyronin B	530/560	0.50–0.60	NP–11	558	0.51	0.41
Alizarin red S	545/575	.....	NAr–6	558	1.16	1.10

*These dyes have their absorption maxima so far into the violet light that it proved impractical, with the visual instrument, to record ratios between points on either side of the maximum, and the two points selected were both therefore on the descending leg of the curve. With the Beckman instrument it is possible to get good readings in the violet part of the spectrum; so it is possible to select new points for the ratios of these dyes, one of which is on either side of the maximum; see Table 2.

TABLE 1. *Concluded.*

Dye	Nat. Formulary standards		Comparative determinations			
	Wave lengths of points selected for ratio	Ratio tolerance	Cert. No. of sample tested	Absorption maximum (λ)	B & L Ratio	Beckman Ratio
Violet and Blue Dyes λ=585–670 mμ						
Methyl violet 2B	575/605	1.30–1.50	NMv–9	588	1.44	1.23
Crystal violet	575/605	1.02–1.10	LC–20	595	1.06	0.92
Thionin	590/620	1.40–1.55	NT–13	608	1.53	1.05
Anilin blue WS	590/620	0.95–1.05	NK–6	610	0.95	0.95
Indigo carmine	590/620	0.78–0.82	NI–8	615	0.81	0.79
Azure C	605/635	1.00–1.12	NAc–3	625	1.11	0.87
Cresyl violet	620/650	1.14–1.21	NW–15	630	1.18	1.08
Brill. cresyl blue	605/635	0.82–0.95	NV–32	632	0.88	0.86
Azure A	620/650	.....	NAz–11	635	1.23	1.05
Toluidine blue O	620/650	0.99–1.39	NU–3	640	0.99	0.87
Nile blue A	620/650	0.69–1.04	NNb–3	640	1.00	0.89
Azure B	635/665	.....	NAb–2	660	0.92	0.81
Methylene blue	635/665	0.56–0.62	LA–13	668	0.61	0.54
Green Dyes λ=620–640 mμ (400–430 mμ)						
Fast green FCF	605/635	0.76–0.83	NGf–8	628 (430)	0.80	0.57
Malachite green	605/635	0.83–0.88	NMg–12	630 (420)	0.87	0.68
Brilliant green	620/650	1.51–1.62	NBg–15	ca. 635 (420)	1.54	1.16
Methyl green	620/650	0.88–1.00	NG–25	640 (420)	0.95	0.90
Janus green B	635/665	0.95–1.14	NJ–8	ca. 635 (400)	1.11	1.04
Light green SFy	620/650	0.96–1.06	NL–8	640 (430)	1.03	0.76
Black Dyes						
Chlorazol black E	590/620	.....	NBc–1	605 (480)	1.05	1.05
Nigrosin	575/605	1.00–1.13	NNi–6	580, 615	1.01	0.99

The narrower the slit, the more nearly monochromatic the beam of light which can be measured. It is obvious that the narrower the slit, the more definitive are the wavelength readings that can be made; but it is equally clear that with the visual type instrument, the sensitivity of the human eye sets a limit to the narrowness which is practical. It proves possible therefore to use a narrower beam of light with the photo-electric instrument than with the visual instrument. With the Beckman spectrophotometer, for instance, a nominal wave length band width of 0.6 to 3.2 mμ can be used in the visual spectrum, the smallest size being selected for each wavelength reading with which the required sensitivity of the phototube can be obtained. With the Bausch and Lomb visual instrument employed in the work here referred to, the band width that proved practical was similar,

but could not be accurately controlled. With the General Electric Recording Spectrophotometer, it may be adjusted automatically for a uniform band width, ordinarily 10 mμ, although good results can be obtained at 8mμ.

When spectrophotometric study of stains was begun the effort was made at first to determine the exact location of the absorption maximum for each sample. With the visual type of instrument then in use, this proved a time-consuming operation and a short-cut was soon devised. It was found that if the extinction coefficient (E) of two points were obtained, one on either side of the maximum, the ratio between these two values of E can be used as a measure of the point of maximum absorption. This method was put into practice because of the ease with which the determinations can be made; and, in drawing up the specifications for stains which are now included in the National Formulary, the limits of tolerance are given for this ratio in the case of each dye.

All these ratios, as previously explained, were computed with the use of the visual type of instrument of Bausch and Lomb manufacture. They proved to check very closely with those obtained using the G. E. Recording Spectrophotometer, but not always with those secured with the Beckman Spectrophotometer which is a non-recording photo-electric instrument. The difference in the results has not yet been explained.

Whatever the explanation of the differences, it has seemed desirable to make a series of graphs for typical samples of all the certified biological stains on a Beckman instrument, if for no other reason than because of the increasing popularity of spectrophotometers of that type. The results thus obtained, in comparison with those secured with the visual type instrument, are proving quite interesting and it is planned to present them in a series of papers. The data so far secured are presented in Tables 1 and 2. Table 1 shows: (1) the National Formulary standards, which indicate the limits of ratios as found in the past, with the visual type of instrument, for satisfactory samples of the various dyes; (2) the ratio found for one particular satisfactory (and typical) sample of each dye, using the visual spectrophotometer; (3) the ratio now found for the same sample of each dye, using the Beckman instrument. It will be seen that in some instances the latter ratio is nearly the same as obtained with the visual type of instrument, but most often it is distinctly different; frequently, it lies entirely outside the tolerance of the old standards. It is clear that if such ratios are still to be used, but the data obtained with the

photo-electric type of instrument, entirely new standards must be adopted for many of the dyes.

In some instances, the points selected in the past for obtaining the absorption ratio were chosen wholly with regard to the limitations of the visual instrument, and it proves that with the photo-electric instrument a more significant ratio can be obtained if the points are located at a slightly different part of the spectrum. This is true particularly in the case of yellow, brown, and orange dyes, as their primary absorption is in, or near, the violet, where accurate visual readings are difficult. Because of this difficulty in recording readings in the violet, the two points formerly selected in the case of brown, orange and yellow dyes were both on the descending leg of the

TABLE 2. RECOMMENDED RATIOS* FOR DYES WITH THEIR ABSORPTION MAXIMA IN THE VIOLET PART OF THE SPECTRUM.

	Wave-lengths of points selected		Observations recorded on Beckman instrument	
	Nat. Formulary standard	New recommendation	Cert. No. of sample tested	Ratio at points of new recommendation
	mμ	mμ		
Auramine O.....	470/490	425/455	NAu–1	1.24
Bismarck brown Y.	500/530	460/490	NN–11	1.10
Martius yellow....	500/520	425/455	NYm–3	0.98
Methyl orange....	500/530	425/455	NM–9	1.01
Orange G.....	500/530	470/510	NO–10	1.03
Orange II.....	500/530	4 0/510	COb–2	0.92

*Although these new ratios are recommended to replace the older ones, it must be recognized that all such ratios may be supplanted in the future by other types of spectral analyses. (See text).

absorption curve instead of one on each side of the maximum as with the dyes of other colors. As photo-electric cells are quite sensitive to violet light, this limitation does not hold with the Beckman spectrophotometer, and it is possible to select points on each side of the maximum, even with a yellow dye, and thus to secure much more significant ratios. The new recommendations for such dyes are given in Table 2.

As a matter of fact, however, it is entirely probable that with the photo-electric spectrophotometers now available, the short-cut involved in the use of these ratios will no longer be necessary. It is possible to find the actual maximum of the curve so much more rapidly on such an instrument than on the older type that it can be

obtained even in the routine examinations of stains, and values of more significance than the absorption ratio can be readily secured. It is planned to give such values for the various stains in the future papers of this series.

Since completing the above article, the authors have had their attention called to the fact that the majority of the dyes in which they found great discrepancy between the ratios as obtained with the two spectrophotometers were either fluorescent dyes or metachromatic stains. Further investigation is needed to show what connection, if any, there is between this dichroism and the discrepancy in the ratios.

THE DIFFERENTIATION OF PLACOID, CTENOID AND CYCLOID SCALES BY MEANS OF ALIZARIN RED S

T. WALLEY WILLIAMS, JR., *Department of Anatomy and Histology, West Virginia University, Morgantown, West Virginia*

ABSTRACT.—**This technic has been used successfully by the author for staining, *in situ*, the placoid scales of selachians and the smaller forms of cycloid and ctenoid scales of teleosts. Sections of skin are dissected from the ventrum of the specimen, cleaned of fascia and muscle tissue and fixed in a 10% formalin solution. The section is macerated in several changes of a 3% KOH solution until translucent. Staining is accomplished in a fresh 2% KOH solution to which is added a saturated alcoholic solution of alizarin red S. The section remains in the stain for 24 hours. If necessary, the tissue may be quickly destained in acid alcohol (1% H_2SO_4 in 95% alcohol). The skin is dehydrated in cellosolve or the alcohol series and cleared in methyl salicylate. Placoid and teleost scales prepared in this manner are stained a brilliant red. The various parts of the denticle are well differentiated.**

INTRODUCTION

The placoid scale or dermal denticle of the selachians is a particularly interesting structure to the comparative anatomist. The concept that this type of scale gave origin, in some manner, to the much modified mammalian tooth seems to be generally accepted today. Though a study of the placoid scale of such forms as *Squalus acanthias* or *Squalus sucklii* is invariably included in a course of general comparative anatomy, the author feels that there has been no satisfactory method evolved for demonstrating this significant structure. The technic usually employed in preparing scales of this type for class use is to corrode the skin in strong (30%) KOH or NaOH so as to completely macerate the soft tissues, allowing the scales to precipitate to the bottom of the container. The scales are then washed, run up through a clearing agent and mounted in balsam. This technic is one recommended by Galigher[1]. Another still less satisfactory method in common use is to clear a small section of skin in glycerin, mounting it finally in glycerin jelly. Neither of these preparations will satisfactorily differentiate the various parts of the placoid scale nor show the

[1]Galigher, A. E. 1934. The Essentials of Practical Microtechnique. Albert E. Galigher, Inc. Laboratory of Microtechnique, Berkeley, Cal. *See p. 107.*

relation of the basal plate to the corium in which it is embedded. The method presented in this paper, primarily for the placoid scale, is also applicable to the small forms of the teleost, ctenoid and cycloid types. The dye used is specific for $CaCO_3$ and has been employed by the author[2] and other workers for the staining of embryonic and adult bone.

Technic

1. *Selection and fixation.* The whole specimen is fixed in 10% formalin for at least 1 week. A large section of the integument is then cut away which later may be trimmed to sizes convenient for mounting. It is best to remove the integument from the ventrum of the specimen where it is comparatively free of concentrations of pigment. All shreds of muscle tissue and superficial fascia are carefully removed from the resected skin.

2. *Maceration.* The skin is macerated in several changes of a 3% solution of KOH for about 72 hours, the length of time varying with the thickness of the tissue. At the end of maceration the skin should appear translucent and white. The scales should remain firmly adherent to the skin and show no evidence of sloughing off. This process may be facilitated by exposure to sunlight or ultra-violet rays.

3. *Staining.* The macerated skin is next transferred to a fresh solution of 2% KOH to which is added, by drops, a saturated alcoholic solution of alizarin red S[3]. Enough of the latter is added to the hydroxide solution to color it a deep red. The skin is left in the staining solution for 24 hours, at the end of which time, if maceration has been sufficient, the scales will be stained intensely, the soft tissues remaining unstained. Any excess stain may be removed quickly by placing the tissue in acid alcohol (1% H_2SO_4 in 95% alcohol).

4. *Dehydration, clearing and mounting.* Dehydration is accomplished by running the tissue through 2 changes of cellosolve[4] of 2 hours each. Those who prefer the alcohol series may substitute with changes of 50%, 80% and 95% alcohol followed by 2 changes of benzene. Though clearing may be accomplished in any of the standard clearing reagents, the author recommends methyl salicylate (synthetic oil of wintergreen). After dehydration the tissue is transferred to solutions of 25%, 50% and 75% of methyl salicylate in cellosolve, for 2 hours each. It then may be placed in pure methyl salicylate for permanent storage. Small sections of the stained and

[2]Williams, T. W. 1941. Alizarin red S and toluidine blue for differentiating adult or embryonic bone and cartilage. Stain Techn., **16**, 23–5.

[3]The dye was from Coleman and Bell, No. 40064 No. 4.

[4]'Cellosolve', manufactured by the Carbide and Chemicals Corporation, N. Y.

Photomicrograph of the integument of *Squalus acanthias*. Soft tissues cleared, placoid scales stained. (×13). This plate is a stereogram and for relationships in three dimensions it may be viewed through a stereoscope.

cleared integument may be mounted on slides in neutral Canada balsam.

The following modification of this technic is suggested for those who might prefer a simplified method for classroom use; it will, however, render the soft tissues more opaque: Omitting step 4, the skin is transferred directly from the KOH-alizarin solution into a series of 50%, 70% and 80% glycerin solutions for 3 hours each. It may then be stored in pure glycerin or mounted in glycerin jelly.

Where possible, it is urged that these preparations be viewed through a binocular dissecting microscope for the added three dimensional effect. The preparation is further enhanced by the use of blue, transmitted light.

Discussion

Selachian placoid scales prepared in the manner described above present a strikingly beautiful appearance and are well differentiated. The denticles are stained a brilliant red in contrast to a white background formed by the cleared integument. The basal plate of the scale, formed of cementum and embedded in the corium, stains very deeply. The extra-integumental spine, composed of dentine capped with enamel, stains less intensely. An added advantage is the fact that the scales remain *in situ*, their relations to one another and to the integument remaining intact.

THE BODIAN TECHNIC AND THE MOSQUITO NERVOUS SYSTEM

WM. M. ROGOFF, *62 Hobson Avenue, East Haven, Connecticut*

ABSTRACT.—**Fixation of mosquitoes with Petrunkevitch's paranitro-phenol fluid with 20 or 25% formalin was found to allow use of Bodian activated protargol technic, where other commonly used fixing fluids yielded no success. Gold toning with subsequent oxalic acid reduction was found to be necessary for Culicid nerve tract preparations.**

In preparing a series of sections for a study of the nerve tracts of the mosquito cephalic ganglia[1], some difficulty was encountered in attempting to use the Bodian activated Protargol technic[2]. This method, which had been developed primarily for vertebrate material, was used successfully with the mosquitoes in question (*Culex pipiens* L., *Culex restuans* Theobald) only after certain modifications of the original technic had been made.

FIXATION

Eleven fixing solutions were tried in an attempt to find a fixative which would permit good preparations with the technic in question. The fixatives used, and their composition, are described in table 1.

Adult mosquitoes fixed in preparations 1 to 6 (cf. table 1) failed to show fiber tracts in the central nervous system after treatment by the Bodian method. Good fiber tract preparations were obtained after use of fixatives 7 to 11. It should be noted that the Petrunkevitch paranitro-phenol fluid containing 10% by volume of formalin was not suitable for demonstrating nerve tracts, while the same fluid containing 20% formalin was quite adequate. Fixatives 9 to 11 allowed too much shrinkage to be acceptable for use despite the staining success. Thus the paranitro-phenol fluid with either 20% or with 25% formalin was used predominantly as the fixing fluid for the preparation of slides for this study.

DEHYDRATION AND CLEARING

Specimens were dehydrated either with a graded series of alcohols, or with dioxane. Xylene was used as the clearing agent.

[1]Rogoff, W. M. 1944. The anatomy and metamorphosis of the cephalic ganglia of the mosquito. Cornell U. Abstracts of Theses, **1943**, 291–3.

[2]Bodian D. 1936. A new method for staining nerve fibers and nerve endings in mounted paraffin sections. Anat. Record, **65**, 89–97.

1937. The staining of paraffin sections of nervous tissues with activated protargol. The role of fixatives. Anat. Record, **69**, 153–62.

Embedding and Sectioning

Almost all specimens were embedded in a rubber-paraffin mixture ("Tissue-mat", marketed by the Fisher Scientific Company), though a few were placed in standard paraffin. It was found that both media sectioned nicely and allowed only a minimum amount of fragmentation of the chitinous cuticula of the mosquitoes. Sections were cut mostly at 10μ.

Table 1. Fixatives and Composition

1. Formol-saline	Formalin	10 ml.
	1% salt solution	90 ml.
2. Petrunkevitch's	Water	60 ml.
Sublimate	Absolute alcohol	40 ml.
	Glacial acetic acid	18 ml.
	Nitric acid	2 ml.
	Mercuric chloride to saturation	
3. Bouin's picro-formol	Picric acid (sat. aq.)	75 ml.
	Formalin	25 ml.
	Acetic acid (glacial)	5 ml.
4. Bouin's picro-formol plus turgitol	Turgitol is a commercial detergent	
5. Davenport & Kline	Trichloracetic acid	10 grams
modified Hoffker's	Glacial acetic acid	10 ml.
trichloracetic	Normal butyl alcohol	60 ml.
(at 70° C.)[3]	Normal propyl alcohol	20 ml.
6. Petrunkevitch's	60% alcohol	100 ml.
paranitro-phenol	Nitric acid	3 ml.
with 10% formalin	Ether	5 ml.
	Cupric nitrate	2 grams
	Paranitro-phenol	5 grams
	Formalin (reagent)	12 ml.
	(Formalin added just before use)	
7. Petrunkevitch's paranitro-phenol 20% formalin		
8. Petrunkevitch's paranitro-phenol 25% formalin		
9. Petrunkevitch's paranitro-phenol 33% formalin		
10. Petrunkevitch's paranitro-phenol 50% formalin		
11. Formol-ether-alcohol	Formalin (reagent)	25 ml.
	Ether	5 ml.
	70% alcohol	70 ml.

[3]Davenport, H., and Kline, C. L. 1938. Experiments with Bodian's method. Stain Techn., 13, 147–58.

Affixing Sections to Slides

Because of the long chemical and incubation treatment, it was necessary to have the glass slides scrupulously clean, before affixing sections, to prevent undue loss. It was found that slides cleaned in the standard K_2CrO_4-H_2SO_4 mixture, rinsed in water, dried, and used immediately were satisfactory for this work. Mayer's albumen mixture was used as the adhesive. The sections were heated just before dissolving out the paraffin prior to staining.

Staining

Preparations were stained with Bodian's silver impregnation technic, involving the use of activated protargol. (Protargol, a brand of strong protein silver, i. e. silver albumose, is marketed by the Winthrop Chemical Company). Details of the staining technic follow:

1. *Impregnation.* Sections are placed in a solution of 1% protargol containing 4-6 grams of metallic copper (copper wire, shavings, sheet, etc.) per 100 ml. of solution, and incubated for 24–48 hours at 37° C. It was found desirable to cleanse the copper with concentrated nitric acid before using. The protargol-copper bath may be used only once. Sections are then washed in distilled water.

2. *Reduction.* Sections are placed in a reducing solution (1 gram hydroquinone, 5 grams sodium sulphite, 100 ml. water) and left for 5 to 10 minutes. They are then washed thoroughly in about three changes of distilled water.

3. *Gold toning.* Gold toning was found necessary for Culicid nervous system preparations. This toning is accomplished as follows:

(a) Sections are placed in a solution of 1% gold chloride, containing about three drops of glacial acetic acid per 100 ml. of solution, until they are decolorized (usually 2 to 5 minutes).

(b) Sections are rinsed rapidly in distilled water—one dip is usually sufficient.

(c) Sections are placed in 2% oxalic acid until they have a faint purplish (or blue) tinge—usually 2 to 5 minutes, after which they are washed in distilled water.

4. *Stabilization.* Residual silver salts are removed in a solution of 5% sodium thiosulfate (hypo) for 5 to 10 minutes, after which the sections are washed thoroughly in distilled water, dehydrated, and mounted in damar or balsam.

IMPROVED FIXATION IN VITALLY STAINED METHYLENE BLUE PREPARATIONS[1]

ELBERT C. COLE, *Thompson Biological Laboratory*
Williams College, Williamstown, Mass.

ABSTRACT.—**The present paper reports that ammonium molybdate dissolved in physiological saline for amphibian and mammalian tissues, and in sea water for squid tissues, forms a fixing solution that greatly reduces cellular distortion in vitally stained permanent methylene blue preparations.**

Permanent preparations of tissues stained vitally with methylene blue generally show considerable cellular distortion. This phenomenon has been attributed to abrupt steps in dehydration. Rapid dehydration is necessary because methylene blue is soluble in alcohol even after fixation with ammonium molybdate, and the loss of even minute quantities of stain may alter the final picture at critical points (Cole, 1925, pp. 384–5). To reduce the loss of dye and to permit a more leisurely rate of dehydration, the writer used two methods (Cole, 1934, 1936). In the second method, where isotonic saline was used as a solvent for the fixing agent, less distortion occurred than in the first method. Re-examination of a large series of preparations indicated that the nature of the fixing solution might have had something to do with the degree of distortion. The writer long ago noted that distortion was somewhat reduced, in the methylene blue technic, if Ringer's solution was used as a solvent for the ammonium molybdate used in fixation (Cole, 1925, p. 377). Subsequent work has shown that physiological saline is similarly effective for amphibian and mammalian tissues.

Carlton (1922) pointed out that the use of isotonic saline in place of distilled water has no effect when used with mercuric chloride as a fixing agent, but that it did considerably reduce distortion when used with formalin in fixation. Similarly, Stowell (1941) in a study on the effects of various methods of fixation, dehydration, and embedding on tissue volume, noted that the use of isotonic diluents slightly improved formalin fixation.

A long series of studies on the integument of the squid, *Loligo, pealii, Les.*, has shown that ammonium molybdate dissolved in sea water gives far less cellular distortion than when the salt is dissolved

[1]This investigation was aided by a grant from the Williams 1900 Fund.

in distilled water. Thus it appears that with improper fixing solutions the major part of the distortion occurs previous to the admittedly harsh but necessary dehydration procedure. That the use of sea water instead of distilled water is also applicable to other fixing agents has been shown by Young (1935). Young showed that both Champy's solution and "4% formaldehyde" caused less distortion in the stellate ganglion of *Sepia* when sea water was used in place of distilled water in preparing the respective fixing solutions. Both Carlton (1922) and Young (1935) demonstrated that the effects varied with different fixing solutions. The latter suggested that possibly the use of sea water (and presumably physiological saline) maintained normal osmotic pressures external to the tissues until the slower moving ions of the fixing solution reached the point where they could balance internal pressures. The present paper reports that ammonium molybdate dissolved in physiological saline for amphibian and mammalian tissues, and in sea water for squid tissues, forms a fixing solution that greatly reduces cellular distortion in vitally stained permanent methylene blue preparations.

REFERENCES

CARLTON, H. M. 1922. Notes on the comparative effects on tissues of isotonic saline and distilled water when used as solvents for mercuric chloride and formol in histological fixation. Quart. J. Micr. Sci., **66**, 501–8.

COLE, ELBERT C. 1925. Anastomosing cells in the myenteric plexus of the frog. J. Comp. Neurol., **38**, 375–87.

————————. 1934. An improved fixing solution for methylene blue preparations. Stain Techn., **9**, 89–90.

————————. 1936. A new methylene blue technic for permanent preparations. Stain Techn., **11**, 45–7.

STOWELL, ROBERT E. 1941. Effect on tissue volume of various methods of fixation, dehydration, and embedding. Stain Techn., **16**, 67–83.

YOUNG, J. Z. 1935. Osmotic pressure of fixing solutions. Nature, **135**, 823–4.

A SIMPLIFIED STAIN FOR HEMOGLOBIN IN TISSUES OR SMEARS USING PATENT BLUE

R. C. DUNN, *Surgeon, U. S. Public Health Service*
With the technical assistance of E. C. THOMPSON, *Assistant Bacteriologist National Institute of Health, Bethesda, Md.*

ABSTRACT.—**The following technic, based on the patent blue V hemoglobin reaction, is useful for identifying hemoglobin in tissue fixed in neutral formaldehyde solution and embedded in paraffin:**

Stain the deparaffinized, hydrated sections 3 to 5 minutes in the working reagent, prepared by adding 2 ml. of glacial acetic acid and 1 ml. of 3% hydrogen peroxide to 10 ml. of the filtered stock solution (1 g. patent blue, 10 g. zinc powder, and 2 ml. glacial acetic acid). Counterstain 30 to 60 seconds in 1:1000 safranin solution in 1% acetic acid, rinse, dehydrate with alcohols, clear in xylene and mount in clarite. Total time required, 37 minutes.

Blood and tissue and smears may be stained, following fixation in methyl alcohol, by applying the working reagent as above.

The basic principles of this staining technic were described by Lison[1], who used it in identifying hemoglobin globules and casts in the kidneys of frogs following intraglomerular injections of hemoglobin solutions. In these amphibia it was apparently necessary for him to fix the delicate hemoglobin globules in Slonimski-Lapinski solution (potassium ferricyanide, 2.5–4 g.; 10–20 ml. of 38% formaldehyde soution; 100 ml. water) and make frozen sections of the tissue. Both these procedures offered obstructions to the routine use of the method and we found that both could be eliminated in our work on chemical hemolysis in warm-blooded animals.

We compared the kidneys of mice, rats and guinea pigs containing numerous freshly formed hemoglobin casts and cytoplasmic globules in the epithelium of the convoluted tubules in the following manner: half of one kidney was fixed in ferricyanide formalin and frozen sections cut; the other half was fixed in 4% formaldehyde solution buffered to pH 7.0, embedded in parraffin, and sections cut. The latter is our routine procedure. The staining qualities of the sections prepared by the two methods were compared; leuco patent blue V was used. The results appeared even more satisfactory by following

[1]Lison, L. 1938. Zur Frage der Ausscheidung und Speicherung des Hämoglobins in der Amphibienniere. Beitr. path. Anat. allg. Path., **101**, 94–108.

our usual fixation and embedding technic than with the procedure recommended for amphibia. This was not our experience with frogs, in which hemoglobin globules stained very poorly without special fixatives.

Because we had in our files numerous animal tissues[2] stained by hematoxylin-picric-acid technic[3] for hemoglobin, we were able to make a close comparison of the results with these two methods. In all cases the results were satisfactory. The patent blue stain did not give the "false positive" reactions of the other stain which always reacts with trichohyalin granules and, occasionally, with dense keratin. Since the patent blue reaction (peroxidase type) is sensitive, the technic must be precise. For example, in tissue fixed in Zenker's fluid the hemoglobin-staining with patent blue is weak, while if Bouin's fluid is used for fixation of tissue the hemoglobin is not stained.

On the basis of this experience, the simplified patent blue technic is recommended as a good, reliable procedure suitable for routine use under the conditions described.

The simplified patent blue stain technic is as follows:

A. Tissue blocks should not be thicker than 3 to 5 mm. They should be fixed about 24 to 28 hours—prolonged fixation should be avoided—in a 4% formaldehyde solution buffered to pH 7.0. For the best results perfect fixation is necessary. The tissue is then embedded in paraffin and sectioned as usual. We find that sections 5 to 6μ thick are the most satisfactory.

B. The patent blue stock solution is prepared as follows:

Patent blue V (CI No. 712, NAC–8137)	1.0 g.
Water, distilled	100.0 ml.

Dissolve the dye in the water, then add:

Zinc powder, C. P.	10.0 g.
Glacial acetic acid	2.0 ml.

After adding the zinc and acetic acid to the dye solution, bring the mixture to a boil. In a short time a complete decolorization (loss of blue color) will occur. This solution is said to be stable.

To prepare the working reagent:

Filter, just before use, 10 ml. of the stock solution and add 2 ml. glacial acetic acid and 1 ml. commercial hydrogen peroxide (3%).

---

[2]From the studies on chemical hemolysis of Dr. Stewart Webster, Biochemist, Industrial Hygiene Research Laboratory, National Institute of Health.

[3]Dunn, R. C., and Thompson E. C. 1945. A new hemoglobin stain for histologic use: a slightly modified Van Gieson stain. Arch Path., 39, 49–50.

C. To prepare carmine alum:

Carmine (alum lake)	1.0 g.
Water, distilled	200.0 ml.
Salicylic acid	0.2 g.

Dissolve the dye in the water with the aid of heat; when cool, add salicylic acid as a preservative and filter.

D. To stain the sections:

1. Bring paraffin sections to water.
2. Stain in the working reagent (freshly prepared), 3 to 5 minutes.
3. Wash briefly in water.
4. Counterstain in carmine alum, 30 to 60 seconds (or use a weak solution of safranin: 0.1 g. safranin, 1 ml. glacial acetic acid, 99 ml. distilled water.)
5. Dehydrate with alcohols, clear in xylene and mount in clarite.

E. Results: Hemoglobin stains dark blue-green, the background a light pink color.

Methyl-alcohol-fixed blood and tissue smears may be stained by applying the working reagent as above. Since the patent blue stain is essentially a peroxidase type of reaction, the granules in certain leucocytes, particularly eosinophils, stain dark blue.

A SIMPLIFIED METHOD OF PREPARATION OF DI-AMMINE-SILVER HYDROXIDE FOR RETICULUM IMPREGNATION; COMMENTS ON THE NATURE OF THE SO-CALLED SENSITIZATION BEFORE IMPREGNATION[1]

R. D. LILLIE, *Medical Director, U. S. Public Health Service, Bethesda, Md.;* with the technical assistance of J. GRECO

ABSTRACT.—**A satisfactory di-ammine-silver hydroxide solution may be repeatedly and consistently prepared by adding 9 or 10 volumes of 10% silver nitrate solution to 1 volume of 28% ammonia water, running in the first 6 or 7 volumes rapidly and proceeding cautiously from then on, shaking until clear after each addition, until a faint permanent turbidity is reached.**

The essential nature of Gomori's iron alum treatment and of Wilder's uranyl nitrate step following the Weigert permanganate-oxalic-acid sequence appears to be an oxidation, since the same results may be achieved with chromic acid, hydrogen peroxide, sodium iodate and elemental iodine, and since this step is better omitted on previously chromated material.

The purpose of this paper is to introduce a variation in the manufacture of the ammoniacal silver oxide solution which, by giving a more definite end-point, makes easier the duplication of the solution. Further exploration was made also of the "sensitizing" bath with uranyl nitrate suggested by Wilder and the bath with ferric ammonium alum suggested by Gomori.

According to the Bielschowski-Maresch technic about 2/3 of the 1.08 g. $AgNO_3$ in 10 ml. of 10% silver nitrate solution is precipitated by addition of 5 drops or about 0.3 ml. of 40% sodium hydroxide solution (equivalent to about 170 mg. NaOH). Then 28% ammonia water is added drop by drop until the precipitate is almost all redissolved. In practice this exact point is difficult to duplicate, and one or two drops of ammonia water seem to make a considerable difference in the action of the solution on tissues,

This procedure is essentially similar in various Bielschowski variants, and the same general method is followed in the del Rio Hortega diammine-silver carbonate variants. Gomori alone has proposed adding more silver nitrate after the silver oxide has been dissolved. He directed adding further silver nitrate until the brown precipitate

[1]From the Pathology Laboratory of the National Institute of Health, Bethesda, Md.

formed dissolves easily on shaking. In practice this is found to be an extremely vague end-point, and Gomori writes me that his actual practice is to continue the addition of silver nitrate until the precipitate dissolves only with difficulty, or even to the point of a faint permanent turbidity, when he then adds 1 more drop of 2% ammonia water to clear.

Being unimpressed with the importance of having potassium or sodium nitrate as well as, or in place of, ammonium nitrate in the final solution, I tried the procedure of placing a measured quantity of ammonia water in a beaker and adding to it 10% silver nitrate solution to the point of faint permanent turbidity. The amounts consumed corresponded quite closely to the reaction $AgNO_3+3NH_4OH \longrightarrow Ag(NH_3)_2OH+2H_2O+NH_4NO_3$. This silver solution worked excellently, using the balance of Gomori's technic. It was at this point that I wrote Gomori asking for an emendation of his directions for preparing the silver solution.

As finally developed our proposed technic follows this sequence.

1. After formalin fixation, run paraffin sections through xylene to 100% alcohol.
2. Immerse in 1% collodion in 100% alcohol and ether, equal parts, 5 to 10 minutes.
3. Drain 1 minute.
4. Place in 80% alcohol, 5 minutes (can wait indefinitely here).
5. Wash in tap water.
6. Apply 0.5% potassium permanganate, 2 minutes.
7. Wash in water.
8. Treat with 5% oxalic acid, 2 minutes.
9. Wash in water.
10. Apply official solution of iron chloride (Liquor Ferri Chloridi U.S.P. XI) 1 ml., distilled water 49 ml., 2 minutes. With Orth fixation omit this step.
11. Wash in tap water, 3 minutes.
12. Wash in distilled water, 2 changes.
13. Immerse in di-ammine-silver hydroxide solution 3 minutes. To prepare: Place 1 ml. 28% ammonia water in a small flask and add 7 or 8 ml. 10% silver nitrate rapidly, then continue to add 10% silver nitrate drop by drop shaking between each addition to clear the solution until a faint permanent turbidity remains after the last drop added. This takes a total of 9 to 10 ml. silver solution. Then dilute the resultant solution with an equal volume of distilled water. This solution can be used for at least two days, but is not used more than once.

Place slides on glass rods face up over a suitable slop jar and drop about 2 ml. of the silver solution on each slide. After 3 minutes

14. Drain and rinse quickly with distilled water.
15. Flood slides with 10% formalin for 2 minutes.
16. Wash in running water 3 minutes.
17. Tone in 0.2% acid gold chloride 2 minutes.
18. Rinse in water.
19. Fix in 5% sodium thiosulphate 2 minutes.
20. Wash in water.
21. Counterstain as desired; e. g., with acetic alum hematoxylin (0.5%) for 2 minutes, followed by washing 2 minutes in water, then with 0.5% eosin or Van Gieson's picro fuchsin for 1 minute, or with an acid fast stain, or with a simple basic anilin dye such as 0.1% aquepus thionin, safranin or toluidine blue.
22. Dehydrate with 95% alcohol after the Van Gieson stain, and then, after all methods, with 3 changes of acetone, which removes the collodion film, and clear with acetone and xylene, equal parts, followed by 2 or 3 changes of xylene.

In this technic the ferric iron mordant before the silver still causes alum hematoxylin to stain nuclei black, and it is sometimes preferable to omit the hematoxylin or possibly to substitute 3% hydrogen peroxide for the ferric salt. Usually a quite complete impregnation of reticulum, and little silvering of other structures are obtained.

On comparative trial it was found that, on formalin-fixed material, omission of the permanganate oxidation of step 6 resulted in failure of impregnation. Omission of the oxalic acid reduction gave rise to a number of failures of impregnation, though some sections showed excellent reticulum.

The ferric chloride (step 10) is necessary for regularity of impregnation of formalin material, though good impregnation may often be obtained without it. In ferric iron content, the ferric chloride corresponds approximately to Gomori's 2% iron alum solution, and was adopted as simpler to prepare. Wilder's 5-to-10-second bath in 1% uranyl nitrate in place of the ferric chloride also gives excellent results.

I must dissent from Wilder's and Gomori's views that this step constitutes a "sensitization" by metal impregnation. Gomori also reported good results with silver, gold, cadium, lead, tin, and uranium salts. He specified ferric salts in the iron group, but did not say whether plumbic and stannic salts were required. Silver and gold salts are readily reducible, and hence can be regarded as oxidants as are ferric and hexavalent uraniun salts. That these salts act as oxi-

dants is suggested by the fact that 3% hydrogen peroxide, 1% sodium iodate, 5% potassium bichromate in 1% acetic acid, and 1% iodine in 2% KI solution could each be substituted for the ferric chloride or uranyl nitrate with quite good to excellent results.

It must be noted that with material primarily fixed in Orth's fluid this step with ferric chloride or other oxidant should be omitted. With this fixation washing with distilled water after the oxalic acid and then silvering give good to excellent results, and introduction of any of the oxidants used successfully with formalin-fixed material tends to engender much precipitate and interfere with the impregnation of reticulum. Only the acetic acid potassium bichromate solution was well tolerated, and it afforded no improvement over direct passage from oxalic acid through water to silver.

The silver solution was tried with good results without adding water. At this concentration 1 minute sufficed. Dilution with an equal volume of water and prolongation of silvering to 2 or 3 minutes seemed to give slightly more consistent results. Higher dilutions gave irregular and inconsistent results in the times tried. Variation of the time in the 50% dilution (1.5, 3 and 6 minutes) made no important difference. However the best time was perhaps 3 minutes.

Various dilutions of strong formalin were tried in step 15. A 1% dilution gave fairly good results with 4 minutes reduction, 2% formalin was irregular in its action, 5% was quite good in general, but there were irregular unsilvered areas in some sections; 10% and 20% formalin for 2 to 4 minutes generally gave good results, while 1 minute was often inadequate.

REFERENCES

MARESCH, R. 1905. Ueber Gitterfasern der Leber und die Verwendbarkeit der Methode Bielschowskys zur Darstellung feinster Bindegewebsfibrillen. Zentbl. allg. Path. u. path. Anat., **16**, 641–9.

WILDER, H. C. 1935. An improved technique for silver impregnation of reticulum fibers. Amer. J. Path., **11**, 817–19.

GOMORI, G. 1937. Silver impregnation of reticulum in paraffin sections. Amer. J. Path., **13**, 993–1002.

THE USE OF CLARITE IN PREPARING WHOLE MICROSCOPIC MOUNTS

ROBERT M. ROSENBAUM, *4730 Centre Ave., Pittsburgh 13, Pa.*

The usual difficulties encountered in the use of glycerin jelly or balsam as the mounting medium in the preparation of whole microscopic mounts can, in a large measure, be overcome by the use of Clarite or Clarite X,[1] both cycloparaffin or napthelene polymers which have found use as histologic mounting media.[2] Several descriptions of such resins for use in microscopy have found their way into the literature lately and their increased popularity as well as the results obtained should prove interesting.[3]

These polymers prove especially useful where microprojection demonstrations are desired or where prolonged microscopic study is necessary. This is due to their high melting point which can stand the heat of an arc lamp very much better than glycerin jelly, Canada balsam or gum damar.

The Clarite whole mount technic is similar to that involved were glycerin jelly used as the mounting medium.[4] For larger specimens some sort of cell is required and the hollow ground slide (cavity slide) similar to those used in the examination of living bacteria has shown distinct advantages. Its greater depth allows specimens as large as 1 mm. to be mounted, while because of the high refractive index of Clarite, no noticeable aberration results. In the case of arthropods, where difficulty is encountered in penetrating the chitin, fixatives of a great penetrating power are used. The sublimate fixatives work well, particularly the Petrunkewitsch modification of Gilson's Fluid[5] which is efficient in regard to its time and end effect on the specimen. The following method has been most successful in the case of *Anoplurida* and *Acarinida*.

---

[1]Courtesy of the Neville Co., Pittsburgh, Pa.

[2]Groat, R. A. 1939. Two new mounting media superior to Canada balsam and gum Damar. Anat. Rec., **74**, 1.

[3]Richards, O. W., *et al.* 1943. Plastic coverglass substitutes in microscopy. *Abs. in* Anat. Rec., **87**, 4.

Para, M. 1944. The application of a processed glue as a substitute for glass coverslips in histologic technique. J. Lab. and Clin. Med., **29**, 1294–9.

[4]Lee, A. B. 1937. The Microtomists Vade-mecum, *10th Ed., edited by J. B. Gatenby.* Blakiston's, Philadelphia. See pp. 223–4.

[5]Gilson Fluid (Petrunkewitsch modification) Water, 300 ml.; glacial acetic acid, 90 ml.; absolute alcohol, 200 ml.; nitric acid, 10 ml.; sublimate to saturation.

To detach any debris which may adhere to the specimen, it is washed in running water over night. The specimen is then killed and fixed in hot Gilson's Fluid (Petrunkewitsch modif.). This should continue for fifteen minutes after which the specimen is washed for one half hour in 70% alcohol. Dehydration is carried out in alcohols of 70%, 80% and 95%, the exposure being thirty minutes for each strength.

Following dehydration the specimen may be kept indefinitely in xylene. At this point staining can be accomplished but caution should be exercised in selection of the schedule since alcoholic stains must be used.

Permanent mounts are made by filling the cavity of the hollow ground slide with a 60% Clarite-xylene solution in excess. The specimen may be manipulated with xylene-dipped needles and thus properly centered. The cover slip is then applied and it will be found that the excess xylene will form a perfect seal around the entire cover slip. Following application of the cover slip, the slide should not be touched for twenty minutes. After this time, the slide may be used normally but care must be taken not to store the slide on its side since, while an air tight seal is formed between the cover slip and the slide, the Clarite in which the specimen is mounted will not be sufficiently hard for at least three weeks and the mounted specimen would become displaced were the slide stored on its side.

TABLE 1.*—COMPARISON OF THE TYPICAL PROPERTIES OF CLARITE AND CLARITE X WITH GUM DAMAR AND CANADA BALSAM

	Clarite	Clarite X	Damar	Canada Balsam
Melting Point C°.	145–155	145–155	105	61
Refractive Index $n_{d_{25}}$	1.544	1.567	1.524	1.535
Color	water white	same	pale amber	dark amber
Effect of Age on Color	stable	stable (yellows slightly)	yellows slowly	yellows rapidly
Acid No.	none	none	32.2	101.3

*Courtesy of the Neville Co., Pittsburgh, Pa. December, 1939.

NOTES ON TECHNIC

Sterile Milk for Background in Capsule Staining

In an article entitled "Staining Bacteria and Yeasts with Acid Dyes" (Stain Technology, **16**, 13, 1941) W. E. Maneval describes a staining solution which contains an acid dye in combination with phenol, ferric chloride, and glacial acetic acid. He suggests its use in the demonstration of capsules, with albumen as a background material.

Sterile litmus milk, however, proves better for the purpose than albumen. In using milk, one places a drop of the sterile fluid on a clean, grease-free slide, and adds to it a small amount of the culture to be tested. The drop is allowed to dry and is then covered with Maneval's Solution A for five minutes. The stain is then washed off, and the smear blotted and dried.

This procedure is simpler than the Huntoon Method, as given in page IV–20 of the Manual of Methods for the Pure Culture Study of Bacteria, and the materials for its use are generally on hand in even the smallest laboratory. The Maneval solution contains not only the dye, but also the fixatives for the casein film.

I have used this method in connection with the study of capsulated bacteria associated with paper mill slime, but have not had the opportunity of trying it on other types of organisms.

Recently an unidentified contaminant has been encountered, in the form of large round globules, the source of which was not determined. It proved, however, that they existed on the surface of the film, and could be wiped off with xylene without disturbing the stained preparation.

Maneval's Stain, when made with more than one dye in the same solution, contains possibilities as a differential poly-stain, as claimed by the author, but I have not been able to determine just how these mixtures work with bacteria. Acid fuchsin and fast green FCF are very good dyes to use, either singly or in combination.—Glen C. Weber, *41 S. Church St., Keyser, West Virginia.*

Further Observations on Ethyl Alcohol Substitutes

A recent paper by the authors (Stain Techn., **20**, 115–17, 1945) reported satisfactory results with various staining solutions made up with isopropanol or methanol in place of ethanol as a solvent. At the time of writing, none of the solutions were more than four

or five months old. At present the solutions have been kept eight to eleven months and a report on their relative keeping qualities can be given.

In this respect there has been essentially no difference between the solvents. All of the carbol fuchsin solutions have decomposed, even those made up with ethyl alcohol. Gelatinization has been observed in the case of carbol crystal violet; but this, as explained elsewhere (Conn, Stain Techn., **21**, 21–2, 1946), is caused by too great concentration of the dye. All the other solutions that have been kept are still good, regardless of the solvent, except that Delafield's hematoxylin made up with methyl alcohol, C.P., Baker, proves distinctly inferior in staining qualities. (The similar solution made up with "Bioloid" anhydrous methyl alcohol, Will, is still perfectly satisfactory.) Accordingly, the conclusions concerning these solvents as expressed in the previous article do not need to be modified after the solutions have aged. There is still no explanation available as to why there is a slight difference in the quality of the solutions made up with the two different grades of methanol.—H. J. Conn and Mary A. Darrow, *Geneva, N. Y.*

LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROSCOPE AND OTHER APPARATUS

ULLRICH, H. **Über die Bedeutung anastigmatischer Beleuchtung für die Polarisationsmikroskopie schwach doppelbrechender Objekte.** *Zts. Wis. Mik.*, **59**, 8–11. 1943.

The author stresses the significance of anastigmatic illumination in polarization microscopy of feebly double refracting objects. Comparison is made in results obtained with the ordinary attachable Nicol prism polarizers and analyzers as used on biological microscopes with the newer filter-polarizers of the "polaroid" type. The latter appear to be very superior in their performance and give better results than some of the older, special mineralogical microscopes.

In addition to the inherent astigmatism produced by the Nicol prisms above mentioned, attention should be given to the source of illumination, i. e., the type of lamp used, whether direct or angular illumination, to eliminate the secondary polarization produced by the mirror. Monochromatic light obviates most of the difficulties but makes observation very tiresome and frequently results in erroneous interpretation.

To obtain best anastigmatic polarization it is recommended that the following rules be observed: 1, illumination should be in the direct axis of the microscope (an opal glass at the light source will improve results if a low voltage filament is used as the light source); 2, maintain a numerical aperture of the illumination slightly less than that of the objective, as far as it is compatible with the desired resolution; 3, avoid the use of oculars which converge considerably; 4, the analyzer should be in the body tube of the microscope. (Note: the disturbances produced in analyzers with Nicol prisms over the eyepiece are always noticeable.)—*J. M. Thuringer.*

WICHTERMAN, RALPH. **A new glass device for staining cover-glass preparations.** *Science*, **103**, 23–4. 1946.

The author describes a glass device which makes possible the staining of ten cover-glass preparations in a single operation without the dangers to the preparations inherent in ordinary methods. The apparatus is fully described with an accompanying figure.—*T. M. McMillion.*

PHOTOMICROGRAPHY

GRÄBNER, K. E. **Graphische Darstellung der Filterfunktionen. Ein Hilfsmittel fur die Mikrofotografie.** *Zts. Wis. Mikr.*, **59**, 12–21. 1943.

The author presents a graphic demonstration of filter function as an aid to photomicrography. Directions are given for the preparation of photographic filters, also a table of the more important filters and filter combinations with their relative exposure factors. The interpretation of the gray scale and the preparation of simplified "Logario-tables" (sensitivity curves) are likewise described.—*J. M. Thuringer.*

HAUSER, E. A., and LE BEAU, D. S. **Light-field ultramicrophotography of lyogels.** *J. Amer. Chem. Soc.*, **68**, 153–4. 1946.

Since color photographs are superior to black and white film for recording fine microstructures, Kodachrome pictures were used as negatives in making enlargements on regular printing paper.—*R. T. Whittenberger.*

MURRAY, NELSON A. **A combined 35 mm. and cut film photomicrographic apparatus.** *J. Techn. Methods*, **24**, 46–50. 1944.

The author describes an inexpensive photomicrographic apparatus which is much more satisfactory than the optical bench apparatus it replaces. Any

camera using a single film holder can be used. The 35 mm. adapter is arranged so that it can be removed from the machine between exposures. The microscope rests on a platform, below which is another platform for the lights. A telescoping back is set with screws so as to insure a sharp image and the adjustment need never be changed. A 10-inch projection distance fills a 3¼ by 4¼ inch frame and at the same time gives an image size corresponding to the magnification indicated by the combination of microscope lenses used.—*Challiss Randall.*

MICROTECHNIC IN GENERAL

KRÜGER, F. **Die Möglichkeit der direkten Überträgung von Objekten aus Alkohol in Paraffin.** *Zoöl. Anz.*, **137**, 226–30. 1942.

The author reports some preliminary attempts to embed tissue in paraffin directly from absolute alcohol. The intermediate steps include the maintenance of a saturated alcoholic atmosphere about the paraffin container together with the addition of absolute alcohol to the melted paraffin. This process of embedding is much slower than the customary methods and the results do not appear to justify the elaborate precautions that must be observed to secure even a moderate degree of success.—*A. B. Dawson.*

DYES AND THEIR BIOLOGICAL USES

BUGYI, BLASIUS. **Weitere Beiträge zur kapillaranalytischen Reinheitsprüfung der histologischen und bakteriologischen Farbstoffe. Zugleich Erwiderung auf die Bemerkungen von Kocsis zu meinem Aufsatz "Reinheitsprüfung der histologischen Farbstoffe".** *Zts. Wis. Mikr.*, **59**, 1–7. 1943.

The author urges the routine use of capillary analytical methods in the examination and periodical checking of stains and chemical reagents in histological and bacteriological laboratories. The methods are based largely upon the chromatographic technic originated by Platz-Neugebauer with modifications introduced by Danckwortt, Cocsis, Meyer and the author. The impression is given that the method is simple, that it may be entrusted to relatively inexperienced laboratory personnel and lastly that none other than the usual equipment on hand in such laboratories is needed. Eventually the author concedes that a Hanovia quartz U.V.L. generator with filters and a Zeiss "Pulffrich" Stuffenphotometer would add greatly to the accuracy of the determinations.

The article is, as indicated in the title, principally polemic in nature giving no details of procedures.—*J. M. Thuringer.*

ANIMAL MICROTECHNIC

MANWELL, REGINALD D. **The J.S.B. stain for blood parasites.** *J. Lab. & Clin. Med.*, **30**, 1078–82. 1945.

The J.S.B. stain (Jaswamt Singh and L. M. Bhattacharji. Indian Med. Gaz., **79**, 102, 1944) is described in detail. The author considers it superior in most respects to the other commonly used processes for staining blood and blood protozoa. Preparations are much like those obtained by the Giemsa method, in definition, clarity and color values, but slightly less resistant to light. The technic is simple and rapid. The solutions are inexpensive, and stable for many months.—*John T. Myers.*

NYKA, W. **A histological study of the lungs of mice infected with typhus rickettsiae.** *J. Path. & Bact.*, **52**, 317–24. 1945.

Rickettsiae in the lungs of mice may be demonstrated by the following procedure: Fix in 10% neutral formol. Stain 30 min.–1 hr. in 1: 10,000 aqueous methyl violet. Differentiate in weak acetic acid (2 drops glacial acetic acid in 100 ml. water) until all cytoplasm is unstained. Counterstain with 1: 10,000 aqueous metanil yellow for a few seconds. Dehydrate with acetone, clear in xylene, and mount in a neutral medium. Rickettsiae stain violet.—*S. H. Hutner.*

PHILLIPS, PAUL H. **A practical method of coloring semen for identification purposes.** *J. Dairy Sci.*, **28**, 843–4. 1945.

A method is described for using a series of stains and dyes to differentiate the semen of various breeds used in the artificial insemination of dairy cattle.

Fresh semen was diluted 1:5 with the fresh egg yolk buffer. Five per cent solutions of methylene blue, Nile blue sulfate, thionin, Janus green, alizarin sulphonate, neutral red and Sudan III were used. Enough of each dye was added, drop by drop, to color the sample. It was impossible to dissolve the Sudan III in aqueous solution and a suspension of this dye was used. The diluted semen samples were stored in the refrigerator at approximately 5° C. Daily microscopic observations were then made on each treated sample until differential motility ratings were established.

Nile blue sulfate, neutral red and Sudan III caused no appreciable detrimental effect upon motility of the semen for several days. Sudan-III-treated samples were superior to yolk buffer alone in maintaining a high degree of vigor and motility. It appeared that the Sudan III through its affinity for free fat and its solution in the fat droplets actually improved the storage medium. This dye was inferior because its insolubility prevented the development of even an average tinge to the diluent. The other dyes tried were not satisfactory, although Janus green could be used for a limited time if necessary.

At the time these observations were made actual breeding tests could not be used as the final and more desirable evaluation.—*William G. Walter.*

VON SCHEVEN, RICHARD. **Ein Beitrag zur Frage der Erythrozytenmembran.** *Zts. Wis. Mikr.*, **59**, 35–9. 1943.

For demonstrating the membrane of erythrocytes, the author proceeds as follows: Add 1 ml. freshly drawn venous blood to 50 ml. of a 0.3% solution of NaCl, agitate and allow to stand for 15 hr. Centrifugalize portions of this in regular centrifuge tube. Pour off supernatant fluid. Add to the sediment an equal amount of fixing and staining fluid prepared as follows: 1 drop of a mixture of equal parts of pyroligneous acid, methyl alcohol and formalin added to 8 drops of the stain (2 ml. Breslau's opal-blue-phloxine-rhodamine, 1 ml. liquified phenol, 17 ml. glycerin, 60 ml. distilled water) and allow to stand 2 hr. after thorough shaking. This fluid does not overstain. To examine, smear a small drop on slide; cover and examine with oil immersion lens. The wrinkled appearance of the erythrocytes is explained by the leached contents of the erythrocytes being partly replaced by the fixing and staining fluid.—*J. M. Thuringer.*

PLANT MICROTECHNIC

MEITRES, MARC. **Heterogeneite structurale de chondrioconte chez Lupinus albus L.** *Compt. Rend. Acad. Sci. Paris.*, **218**, 241–3. 1944.

Chondriosomes are not homogeneous structures but are actually composed of chains of small granules. To demonstrate this structure, root tips are killed by the IVA technic of Regaud (see Meitres, C. R. Soc. Biol., **137**, 225, 1943), with an added wash in a fat solvent between the treatment with formol and bichromate. The detailed fixing technic is as follows: Root tips are placed in 20% neutral formol for 6 days; then transferred for 72 hours to a solution made by mixing 10 ml. water, 20 ml. 96% alcohol, and 100 ml. benzene,—this is shaken and the aqueous phase discarded; a wash of 30 minutes is followed by a post-chromatization according to the technic of Dietrich-Parat.—*T. E. Weier.*

MICROÖRGANISMS

CLANCY, CARL F., and WOLFE, DON M. **A rapid staining method for Rickettsia orientalis.** *Science*, **102**, 483. 1945.

The method for staining *Rickettsia orientalis* in smears from animal tissues and providing differentiation from the tissue background is as follows: Smears of infected yolk sac membranes or other tissues are prepared, air dried, and fixed by heat. The slide is flooded with xylene, drained, and after drying in a current of air, is immersed for 5 min. in a distilled water solution of methylene blue 1:5,000 and basic fuchsin 1:5,000. The preparation is then washed in tap water, dried and examined. The dilute stain is readily prepared from 1% stock solutions of each of the two dyes and should be prepared daily to obtain best results. Smears thus stained show *R. orientalis* as a blue organism on a pinkish purple background.—*T. M. McMillion.*

JASMIN, A. M. **An improved staining method for demonstrating bacterial capsules, with particular reference to Pasteurella.** *J. Bact.*, **50**, 361–3. 1945.

To demonstrate capsules on cells of *Pasteurella mastitidis* and related forms, the following modification of Hiss's method was useful: Transfer the amount of culture picked up on a fine straight platinum wire from the surface of a culture to a loopful of physiological saline containing 0.5 to 1% phenol and 10% blood serum; spread into a thin film on a clean polished slide; fix the dried film with a rapid dip into methyl alcohol; drain and flame to burn off excess alcohol; stain 30 sec. to 1 min. in any common bacterial stain; wash with water. The capsules appear as clear areas against a lightly stained background while the bacteria are darkly stained.—*Virgene Kavanagh.*

ROGERS, T. H. **The inhibition of sulfate-reducing bacteria by dyestuffs.** Part II. **Practical applications in cable storage tanks and gas holders.** (Appendix by E. C. Barton-Wright on application in a flour mill.) *J. Soc. Chem. Ind.*, **64**, 292–5. 1945.

A previous investigation showed that sulfate-reducing bacteria could be inhibited by dyestuffs of the flavine type. This paper describes the application of one of these dyes in preventing the corrosion of submarine cables and the fouling of gas holder waters and a wheat washing plant by bacteria producing H_2S. Dye No. 914, a derivative of 3, 6-diaminoacridine prepared by Imperial Chemical Industries, Ltd., was used usually at a concentration of 1 part in 250,000.—*R. T. Whittenberger.*

HISTOCHEMISTRY

GULBRANSEN, R., PHELPS, R. T., and LANGER, A. **A use of the electron microscope in chemical microscopy.** *Ind. Eng. Chem.*, Anal. Ed., **17**, 646–52. 1945.

Possibilities that the electron microscope may offer in chemical microscopy are discussed and a technic for adapting the microscope to this study is presented. The technic for the controlled growth of crystals on thin plastic films is based on the diffusion of ions and molecules through the film and subsequent precipitation on the film.—*R. T. Whittenberger.*